## REMARKS

Upon entry of this amendment, claims 1, 17, 24 and 26-40 will be pending in the present application. In this amendment, applicants have amended claims 1, 17 and 24 without prejudice to applicants' right to present claims corresponding in scope to the claims pending in the application prior to this amendment in a timely filed continuing application. Further, claims 26-40 have been added. Support for all of the claim amendments is found in the original claim set and in Example 1. Support for the hydroxocobalamin limitation can be found at page 17 and Example 1, at page 61; support for the *E. coli* promoter language is found at page 28 and Example 1.

The application has been amended to correct a few minor typographical errors and further as discussed below with respect to the Figures and sequence listing. No new matter has been added.

Invention

The present invention relates to methods and reagents for altering the metabolism of a cell by genetic engineering to enable the cell to produce a metabolite useful in the synthesis of another product that is not naturally produced in the cell or is produced only in low amounts. The invention is exemplified in the specification with respect to the production of S-methylmalonyl CoA, a substrate used in polyketide biosynthesis, in *E. coli*, a host cell that does not naturally make either S-methylmalonyl CoA or polyketides. The Examiner rejected the application on a number of grounds, and to expedite allowance of claims drawn to important subject matter, the applicants have amended the claims, without prejudice to the applicants' rights to present claims of scope equal to those pending prior to this amendment, to focus the claims on recombinant E. coli host cells that produce S-methylmalonyl CoA and a polyketide that requires S-methylmalonyl Co for its synthesis due to the introduction of either the *Propionibacterium shermanii* methylmalonyl CoA mutase and epimerase genes or the *Streptomyces cinnamonensis* methylmalonyl CoA mutase and *Propionibacterium shermanii* epimerase genes. These host cells have been demonstrated by the applicants to produce S-methylmalonyl CoA and so can be used for heterologous production of polyketides by modular polyketide synthase enzymes that utilize S-methylmalonyl CoA as a starter or extender unit.

Information Disclosure Statement

Applicants gratefully acknowledge the Examiner's indication that two of the three information disclosure statements have been received and the references considered. In addition, applicants filed an information disclosure statement on August 13, 2001, a copy of which is enclosed herewith along with the stamped postcard. Review and consideration of this information disclosure statement is requested as well.

Priority

The Office objects to the specification for containing incorrect priority data in the first lines of the application, and states that priority has not been granted for 60/161,414 because this case was filed greater than one year before the filing date of the present application. Applicants hereby submit a replacement declaration that shows that priority is claimed only to the application having. U.S. Pat. Appl. Ser. No. 60/161,703. Applicants inadvertently submitted a declaration with the incorrect priority information, but intended rather only to show that the present application was *related* to U.S. Pat. Appl. Ser. Nos. 60/161,703 and 60/206,082, as indicated in the first paragraph of the application. In a conference of March 29, 2002, the Examiner confirmed that she examined the application with this understanding making it unnecessary to send out a supplemental Office action. A new declaration will be submitted shortly that makes this correction.

Sequence Listing

The Specification has been amended to be in compliance with the Sequence Rules set forth in 37 C.F.R. § 1.821-1.825. More particularly, sequence identification numbers which were omitted at the time of filing were included on page 67 of the Specification.

The undersigned hereby states that the computer readable form copy (CFR copy) of the substitute Sequence Listing and the paper copy of the substitute Sequence Listing, submitted in accordance with 37 C.F.R. § 1.825(a) and (b), respectively, are the same and contain no new matter. Accordingly, entry of the substitute Sequence Listing into the above-captioned case is respectfully requested.

Drawings

To obviate the objection to the specification for containing figures and/or graphs embedded within the text, applicants eliminated figures and charts presented on pages 58, 59, 62, 74, 82, 83, 84, and 87 from the text of the specification and placed them as Figures 2-9. A corresponding amendment has been made to the Description of the Figures part of the specification to include the description of Figures 2-9.

Abstract

A revised abstract is attached herewith that defines the invention more thoroughly, and thus, this objection is respectfully submitted to be overcome.

Claim Objections

The objections to claims 8, 14, 19 and 20 have been rendered moot by the cancellation of these claims.

The Rejections Under 35 U.S.C. § 112, Second Paragraph

The rejections of claims 1-25 under 35 U.S.C. § 112, second paragraph based on various grounds is traversed and reconsideration is respectfully requested.

With regard to the rejection of claims 1-5 with respect to "precursor required for biosynthesis of the product," this language has either been deleted from the claim as in claim 1 or the claim that contains this language has been deleted, as in claim 2.

Claims 2-16, 1-23 and 25 have been deleted. Amended claims 1, 17 and 24 and new claims 26-40 do not include the terms "substantially," "primary metabolite," "modular, interative, or fungal," "PKS" (undefined), "derived from," "modified to overexpress" and "the birA gene" and thus the terms no longer are at issue.

Accordingly, it is believed this basis for rejection may be withdrawn.

The Rejections Under 35 U.S.C. § 112, First Paragraph (Written Description)

The rejection of claims 1-25 under 35 U.S.C. § 112, first paragraph (written description) is traversed on two grounds. First, a *prima facie* case of has not been established. The burden is

initially on the Office to factually demonstrate why one having ordinary skill in the art would not recognize that applicants were in possession of the concept of the invention as now claimed. Second, the claims comply with the written description requirement as discussed below with respect to the Interim Written Description Guidelines and *Regents of the University of California v. Eli Lilly*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997). Nonetheless, the claims have been amended to include any reference to "functional" phrases to which the Examiner objects. In addition, claim 1 now refers to known mutase and epimerase genes.

According to the specification on page 15, first full paragraph:

> A suitable methylmalonyl CoA mutase (5.4.99.2) gene can be isolated from *Streptomyces cinnamonensis*. See Birch *et al*., 1993, *J. Bacteriol. 175*: 3511-3519 .... Another suitable methylmalonyl CoA mutase gene can be isolated from *Propionibacterium shermanii*. See Marsh *et al*., 1989, *Biochem. J. 260*: 345-352....

With respect to the epimerase, page 67 discloses the epimerase sequence from *Propionibactrerium shermanii*.

In *University of California v. Eli Lilly,* cited by the Office, it is stated that "naming a type of material generally known to exist, *in the absence of knowledge as to what the material consists of,* is not a description of that material (emphasis added)." Here, in contrast, there is sufficient disclosure of what the material in question consists of, for example, sequences disclosed in the cited references or in the present application, so it follows that sufficient description is provided.

Thus, it is respectfully submitted that the applicants were in possession of the claimed invention, and thus, withdrawal of this rejection is respectfully requested.

The Rejections Under 35 U.S.C. § 112, First Paragraph (Enablement)

The rejection of claims 1-25 under 35 U.S.C. § 112, first paragraph (enablement) is traversed and reconsideration is respectfully requested. Applicants gratefully acknowledge the Examiner's indication that particular host cells comprising vectors encoding modular PKS enzymes to make polyketides are enabled. Claim 1 has been so amended to include these aspects and also to make reference to specific enzymes (mutase and epimerase) and a specific product (S-methylmalonyl CoA). It is respectfully submitted that the amended claim 1 and those claims dependent thereon comply with the enablement requirement.

The Rejections Over the Art

The rejections under 35 U.S.C. § 102(b) of a) claims 1-2 and 4-5 over Kealey, b) claims 2-3 over Tuchman and the rejections under 35 U.S.C. § 103 of a) claims 1-2 and 4-6 over Stassi in view of Kao, b) claims 7-8 over Stassi and Birch in view of Kao, c) claims 1-2 and 4-13 over Stassi and Birch in view of Barr, d) claims 14-17 over Stassi and Birch in view of Barr and in further view of McKie, and e) claims 18 and 24-25 over Stassi and Birch in view of Donadio and in view of Barr, and f) claim 21 over Stassi and Birch in view of Barr, are traversed and reconsideration is respectfully requested. Claims 2-5 have been deleted, so the rejection as to these claims is moot. Applicants have amended claim 1 such that these references no longer apply to the subject matter of this claim. For example, the subject matter of claim 19, which was not rejected under §§102 or 103, has been included in amended claim 1. The remainder of the amended or new claims all ultimately depend from claim 1. Withdrawal of these rejections, therefore, is respectfully requested.

Conclusion

The claims have been amended in order to place them in condition for allowance. Such action is respectfully requested.

Respectfully submitted,

Dated: May 14, 2002 By: ______________________

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## EXHIBIT A. VERSION WITH MARKINGS TO SHOW CHANGES MADE

**In the Specification**:

The following description of Figures 2-9 to the Brief Description of the Figures on page 6, line 15, has been added:

Figure 2 shows the construction of pSK – MUT, in which four PCR fragments were sequenced and pieced together to form the complete mutase gene in pSK-bluescript.

Figure 3 shows acyl-CoA analysis in BL21 (DE3) panD strains in vivo.

Figure 4 shows the results of CoA analysis of *E. coli* overexpressing methylmalonyl-CoA mutase. The levels of $^3H$ detected in fractions collected from HPLC of cell-free extracts from $^3H$ ß-alanine-fed *E. coli* harboring either the pET control vector grown without hydroxocobalamin (black trace), pET grown with hydroxocobalamin (blue trace), pET overexpressing the mutase and grown without hydroxocobalamin (green trace), or pET overexpressing the mutase and grown with hydroxocobalamin (red trace) are shown.

Figure 5 shows the three routes or biosynthetic pathways for the synthesis of methylmalonyl-CoA that can be engineered into yeast.

Figure 6 shows acyl-CoA analysis of *E. coli* overexpressing methylmalonyl-CoA mutase. The level of $^3H$ detected in fractions collected from HPLC of cell-free extracts from [$^3H$] ß-alanine-fed *E. coli* harboring either the pET control vector (solid trace) or pET overexpressing the mutase (dashed trace) is shown.

Figure 7 shows acyl-CoA analysis in *S. cerevisiae*. The level of $^3H$ detected in fractions collected from HPLC of cell-free extracts from [$^3H$] ß-alanine-fed *S. cerevisiae* after growth for 24 hours (solid trace), 48 hours (dashed trace) and 66 hours (dotted trace) is shown.

Figure 8 shows common cloning cassette.

Figure 9 shows a general method for cloning genes into yeast expression vectors.

The paragraph, beginning at page 9, line 1, has been amended as follows:

Each of the three polypeptide subunits of DEBS (DEBSI, DEBSII, and DEBSIII) contains 2 extender modules, DEBSI [additionaly] additionally contains the loading module. Collectively, these proteins catalyze the condensation and appropriate reduction of 1 propionyl

CoA starter unit and 6 methylmalonyl CoA extender units. Modules 1, 2, 5, and 6 contain KR domains; module 4 contains a complete set, KR/DH/ER, of reductive and dehydratase domains; and module 3 contains no functional reductive domain. Following the condensation and appropriate dehydration and reduction reactions, the enzyme bound intermediate is lactonized by the TE at the end of extender module 6 to form 6-dEB.

The paragraph, beginning at page 19, line 28, has been amended as follows:

A suitable propionyl CoA carboxylase (6.4.1.3) gene for purposes of the present invention can be isolated from *Streptomyces coelicolor* as reported in GenBank locus AF113605 (pccB), AF113604 (accA2) and AF113603 (accA1) by H. C. Gramajo and colleagues. The propionyl CoA carboxylase gene product requires biotin for activity. If the host cell does not make biotin, then the genes for biotin tranport can be transferred to the host cell. Even if the host cell makes or transports biotin, the endogenous biotin transferase enzyme may not have sufficient activity (whether due to specificity constraints or other reasons) to biotinylate the propionyl CoA carboxylase at the rate required for high level precursor synthesis. In this event, one can simply provide the host cell with a sufficiently active biotin transferase enzyme gene, or if there is an [endongenous] <u>endogenous</u> transferase gene, such as the *birA* gene in *E. coli*, one can simply overexpress that gene by recombinant methods. Many additional genes coding for propionyl CoA carboxylases, or acetyl CoA carboxylases with relaxed substrate specificity that includes propionate, have been reported and can be used as sources for this gene, as shown in the following table.

The paragraph, beginning on page 34, line 20, has been amended as follows:

Methods for generating libraries of polyketides have been greatly improved by cloning PKS genes as a set of three or more mutually selectable plasmids, each carrying a different wild-type or mutant PKS gene, then introducing all possible combinations of the plasmids with wild-type, mutant, and hybrid PKS coding sequences into the same host (see U.S. patent application Serial No. 60/129,731, filed 16 Apr. 1999, and PCT Pub. No. 98/27203, each of which is incorporated herein by reference). This method can also incorporate the use of a KS1° mutant, which by mutational biosynthesis can produce polyketides made from diketide starter units (see Jacobsen *et al.*, 1997, *Science* **277**, 367-369, incorporated herein by reference), as well as the use

of a truncated gene that leads to 12-membered macrolides or an elongated gene that leads to 16-membered ketolides. Moreover, by [utlizing] utilizing in addition one or more vectors that encode glycosyl biosynthesis and transfer genes, such as those of the present invention for megosamine, desosamine, oleandrose, cladinose, and/or mycarose (in any combination), a large collection of glycosylated polyketides can be prepared.

The paragraph, beginning at page 46, line 18, has been amended as follows:

As noted above, the hybrid PKS genes of the invention can be expressed in a host cell that contains the desosamine, megosamine, and/or mycarose biosynthetic genes and corresponding transferase genes as well as the required hydroxylase gene(s), which may be either *picK*, *megK*, or *eryK* (for the C-12 position) and/or *megF* [or*eryF*] or *eryF* (for the C-6 position). The resulting compounds have antibiotic activity but can be further modified, as described in the patent publications referenced above, to yield a desired compound with improved or otherwise desired properties. Alternatively, the aglycone compounds can be produced in the recombinant host cell, and the desired glycosylation and hydroxylation steps carried out *in vitro* or *in vivo*, in the latter case by supplying the converting cell with the aglycone, as described above.

The figures and graphs on pages 58, 59, 62, 74, 82, 83, 84, and 87 have been deleted.

The paragraph, beginning at page 58, line 1, has been amended as follows:

[The schematic below] Figure 2 shows the construction of pSK – MUT, in which four PCR fragments were sequenced and pieced together to form the complete mutase gene in pSK-bluescript.

The paragraph, beginning at page 62, line 2, has been amended as follows:

[The graph above] Figure 4 shows the results of CoA analysis of *E. coli* overexpressing methylmalonyl-CoA mutase. The levels of $^{3}$H detected in fractions collected from HPLC of cell-free extracts from $^{3}$H ß-alanine-fed *E. coli* harboring either the pET control vector grown without hydroxocobalamin (black trace), pET grown with hydroxocobalamin (blue trace), pET overexpressing the mutase and grown without hydroxocobalamin (green trace), or pET overexpressing the mutase and grown with hydroxocobalamin (red trace) are shown.

The paragraph, beginning at page 66, line 28, has been amended as follows:

The sequence of the putative epimerase gene contained in cosmid 117-167-A7 was aligned to the N-terminal epimerase sequence already known. The several hundred base pairs downstream of this sequence were translated in all three frames and a stop codon in one of the frames was found that yielded a protein of the expected size. The entire sequence was used to search the protein database via BLAST analysis, and the sequence showed high homology to the sequence of a putative epimerase from *S. coelicolor* identified in accordance with the methods of the invention. PCR primers were designed based on the DNA sequence of the cloned P. shermanii epimerase and the gene was amplified from *P. shermanii* genomic DNA with *Nde*I and *Bam*HI sites at the 5'-end, an internal *Nde*I site was destroyed near the 5' end, and *Nhe*I and *Avr*II sites were introduced at the 3'-end. Following PCR, the 447 bp product was cloned into a Bluescript vector (143-6-11) and sequenced. Also, four additional sequencing primers were designed to provide several-fold coverage of the epimerase gene. The full epimerase gene sequence provided in isolated and recombinant form by the present invention is shown below <u>(SEQ ID NOS: 1 and 2)</u>.

The paragraph, beginning at page 74, line 8, has been amended as follows:

There are three routes or biosynthetic pathways for the synthesis of methylmalonyl-CoA that can be engineered into yeast, [as shown in the schematic below] <u>as shown in Figure 5</u>. These pathways have been shown to produce methylmalonyl-CoA in *E. coli* and can be used to produce methylmalonyl-CoA in yeast. This example describes the identification of a system for methylmalonyl-CoA production in yeast, and a method for introducing it into the yeast chromosome.

The paragraph, beginning at page 82, line 6, has been amended as follows:

[The graph above] <u>Figure 6</u> shows acyl-CoA analysis of *E. coli* overexpressing methylmalonyl-CoA mutase. The level of $^3$H detected in fractions collected from HPLC of cell-free extracts from [$^3$H] ß-alanine-fed *E. coli* harboring either the pET control vector (solid trace) or pET overexpressing the mutase (dashed trace) is shown.

The paragraph, beginning at page 83, line 12, has been amended as follows:

[The graph above] Figure 7 shows acyl-CoA analysis in *S. cerevisiae*. The level of $^3H$ detected in fractions collected from HPLC of cell-free extracts from [$^3H$] ß-alanine-fed *S. cerevisiae* after growth for 24 hours (solid trace), 48 hours (dashed trace) and 66 hours (dotted trace) is shown. The yeast strain InvSc1 [3], grown in synthetic YNB media lacking pantothenate and ß-alanine, was used for acyl-CoA analysis. Yeast cultures starved of ß-alanine were fed [$^3H$] ß-alanine and the cultures were grown for 24, 48 and 66 hours at 30°C. Cells were disrupted with glass beads in the presence of 10% cold TCA and acyl-CoAs were separated by HPLC and quantified by scintillation counting. The yeast CoA pools were labeled with [$^3H$], but the extent of isotope dilution remains unclear. One can measure the specific activity of total CoA in these strains to ascertain the extent of isotope dilution.

The paragraph, beginning at page 84, line 5, has been amended as follows:

For PKS genes and initial studies of metabolic pathway genes, one can employ the analogous sets of bluescript cloning vectors and yeast 2 micron replicating shuttle vectors used in 6-MSA production [3]. With these vectors, yeast expression is driven by the alcohol dehydrogenase 2 (ADH2) promoter, which is tightly repressed by glucose and is highly active following glucose depletion that occurs after the culture reaches high density. Both vector sets have a "common cloning cassette" that contains, from 5' to 3', a polylinker (L1), the ADH2 (or other) promoter, a *Nde* I restriction site, a polylinker (L2), an ADH2 (or other) terminator, and a polylinker (L3). Due to excess restriction sites in the yeast shuttle vectors, genes of interest are first introduced into intermediate bluescript cloning vectors via the *Nde* I site, to generate the ATG start codon, and a downstream restriction site in the L2 polylinker that is common to the bluescript and yeast shuttle vectors [shown below] (Figure 8). The promoter-gene cassette is then excised as an L1-L2 fragment and transferred to the yeast expression vector containing the transcriptional terminator.

The paragraph, beginning at page 87, line 1, has been amended as follows:

[The schematic above] Figure 9 shows a general method for cloning genes into yeast expression vectors.

**In the Claims:**

1. (Amended) A recombinant E. coli host cell comprising one or more expression vectors that [drive expression of enzymes capable of making a product and a precursor] comprise

a methylmalonyl CoA mutase gene selected from the group consisting of a *Propionibacterium shermanii* methylmalonyl CoA mutase gene and a *Streptomyces cinnamonensis* methylmalonyl CoA mutase gene, and

a *Propionibacterium shermanii* epimerase gene,

wherein said genes produce enzymes capable of making S-methylmalonyl CoA required for biosynthesis of [the product] a polyketide produced by a modular polyketide synthase (PKS) produced by a PKS gene or genes in said host cell,

wherein said host cell, in the absence of said expression vectors, is unable to make said [product] polyketide due to lacking all or a part of a biosynthetic pathway required to produce [the precursor] S-methylmalonyl CoA.

17. (Amended) The host cell of Claim [14] 1 in media that [facilitates B12 uptake] contains hydroxocobalamin.

24. (Amended) An *E. coli* host cell that expresses

[heterologous] a methylmalonyl CoA mutase gene selected from the group consisting of a *Propionibacterium shermanii* methylmalonyl CoA mutase gene and a *Streptomyces cinnamonensis* methylmalonyl CoA mutase gene,

a *Propionibacterium shermanii* epimerase gene[s],

wherein said genes produce enzymes capable of making S-methylmalonyl CoA, and

a modular polyketide synthase (PKS) gene.

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| Docket No.: 30062-20049.00 | Atty: Carolyn Favorito |
| Serial No.: 09/699,136 | Filing Date: October 27, 2000 |
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| **Date of Mailing: August 13, 2001, via First Class Mail** | |

**Papers enclosed herewith:**

1. Information Disclosure Statement (3 pages);
2. PTO form 1449 (1 page);
3. References (1);
4. Return postcard